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Two Analytical Methods for the Measurement of 2,4-D in Oranges: an ELISA Screening Procedure and a GC-MS Confirmatory Procedure*

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Abstract: Two analytical methods are presented for the determination of 2,4-D in oranges.

The screening procedure uses a commercial ELISA kit, designed for the determination of 2,4-D in water. Good semi-quantitative screening data were obtained provided that the standard solutions used to construct the calibration curve were prepared in blank orange extract. The limited quantification range of the kit was due to the narrow linear range and differences in response to 2,4-D when present as the free acid and as various esters.

A GC-MS confirmatory technique involved extraction of 2,4-D from oranges by homogenisation with methanol, filtration and esterification/transesterification with boron trifluoride-methanol. Recoveries for three spiking levels (0·2, 1·0 and 2·0 mg kg^{-1}) for 2,4-D and 2,4-D isopropyl ester were within the range 75–120%.

In a small survey of retail oranges, 10 samples were screened using the ELISA kit and four were found to have $>0{\cdot}2$ mg 2,4-D kg^{-1}. All four residues were confirmed by GC-MS. The ELISA procedure involves approximately half the staff effort of the GC-MS procedure.

Key words: 2,4-D, ELISA, GC-MS, oranges, residues analysis

1 INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) is a systemic herbicide and is also used to prevent pre-harvest drop in citrus fruits. It can be applied as an aqueous solution of a salt or as an oil emulsion of an ester. Published methods for the analysis of 2,4-D residues are complex and time-consuming and typically involve extraction, clean-up, hydrolysis of all forms to the parent compound, derivatisation and determination by gas chromatography with electron capture (GC-ECD) or mass spectrometric (GC-MS) detectors.[1–3]

Enzyme-linked immunosorbent assay (ELISA) kits are available commercially for the analysis of 2,4-D, and a number of other pesticides, in water. Previous work[4] at this laboratory has demonstrated that some of these kits can, with modification, be applied to the analysis of food extracts. The first objective of the present work was to investigate the application of ELISA to the determination of 2,4-D, its salts and esters in oranges.

ELISAs, when used to determine pesticide residues in food extracts, can, at best, be regarded as semi-quantitative screening methods. Potentially reportable residues must always be confirmed by other methods.

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Ideally, mass spectrometry (MS) should be used to provide unequivocal identification of the residue. Thus the second objective of this work was to develop a simple gas chromatography-mass spectrometry (GC-MS) method.

2 MATERIALS AND METHODS

2.1 Materials

2,4-D, its sodium salt, its ethyl and isopropyl esters and 2,4,5-T were purchased from Promochem (Welwyn Garden City, UK). 4-Chlorophenoxyacetic acid (4-CPA) was purchased from Aldrich (Gillingham, UK). Solvents were glass distilled (GD) or HPLC grade from Rathburns (Walkerburn, UK). Celite was from Koch-Light (Haverhill, UK).

The ELISA kits were purchased from Millipore (Bedford, MA, USA) and consisted of 96-well microplates.

Organically grown oranges were used for the method development. Samples (non-organically grown oranges) were purchased from retail outlets.

2.2 Sample preparation and extraction

Oranges (including peel) were homogenised in a food processor. The resulting homogenate was stored in polyethylene bags at 2–6°C (<1 week) or −18 to −20°C (>1 week).

For ELISA, orange homogenate (5 g) was combined with celite (10 g) and methanol (50 ml) in a wide-necked polypropylene bottle (250 ml) and blended (4 min) using an Ultra-Turrax homogeniser. The blended sample was filtered through a sintered-glass Büchner funnel under reduced pressure. The homogeniser and bottle were rinsed with methanol (20 ml) and the combined extracts transferred to a volumetric flask (100 ml) and made up to volume with methanol. This extract was then diluted with water (1 + 9 by volume).

For GC-MS, orange homogenate (20 g) was combined with celite (10 g) and methanol (125 ml) in a wide necked polypropylene bottle (250 ml) and blended (5 min) using an Ultra-Turrax homogeniser. The blended sample was filtered through a sintered-glass Büchner funnel under reduced pressure. The homogeniser and bottle were rinsed with methanol (20 ml) and the rinsings passed through the filter cake before further washing of the filter cake (methanol; 2 × 20 ml). The filtrate was transferred quantitatively to a volumetric flask (200 ml) and made up to volume with methanol. This extract was then diluted with water (1 + 9 by volume).

2.3 ELISA

The kits were stored at 2–6°C and equilibrated at room temperature for 30 min prior to use. Determinations were made in duplicate and all manipulations were made in the same order across the plate.

Diluted sample extract (as in Section 2.2) or standard blank orange extract (150 μl) were added to each well followed by 2,4-D enzyme conjugate reagent (50 μl). The contents were gently mixed by swirling on the bench top (1 min). The wells were covered with tape and the plate incubated at room temperature on an orbital shaker (1 h). The well contents were then shaken out to waste and the wells rinsed five times with cool running tap water to remove all unbound conjugate. After the final rinse, the inverted wells were tapped repeatedly onto a paper towel to remove residual water. One hundred microlitres of the chromogenic substrate was added to each well and the microplate re-covered with fresh tape and incubated for a further 30 min on the orbital shaker. The tape was then carefully removed and the stop solution (1 M hydrochloric acid; 100 μl) was added to each well. The contents were mixed, placed into the plate reader, the absorbances measured at 450 nm and the mean of the two determinations for each sample/standard calculated.

2.4 Chromatographic method

2.4.1 Methylation

Following extraction (as in Section 2.2), 2,4-D residues (both acid and esters) were converted to the methyl ester as follows: sample extract (10 ml) was pipetted into a glass flask (50 ml) and 4-chlorophenoxyacetic acid in methanol (4-CPA; 1 μg ml^{-1}; 100 μl) internal standard was added, followed by boron trifluoride-methanol reagent (5 ml, $BF_3 \sim 500$ g $litre^{-1}$). A glass stopper was fitted loosely and the flask was heated in an oven at 110°C for 10 min. The reaction mixture was allowed to stand at room temperature overnight, before being transferred quantitatively to a separating funnel (100 ml) containing hexane (20 ml). The funnel was tightly stoppered and shaken vigorously for 2 min (releasing any excess pressure at intervals). Saturated aqueous sodium chloride solution (10 ml) was added and the funnel shaken for a further minute. The lower aqueous layer was run off into a beaker and the hexane layer run into a second beaker: as some sodium chloride was precipitated during the extraction it was found helpful to add a few ml of water to the funnel and shake for 30 s to dissolve the precipitated sodium chloride and thus avoid the tap of the funnel becoming blocked. The aqueous layer was returned quantitatively to the separating funnel and re-extracted with hexane (15 ml) before being discarded and the two hexane extracts were combined in the funnel. The combined extract was

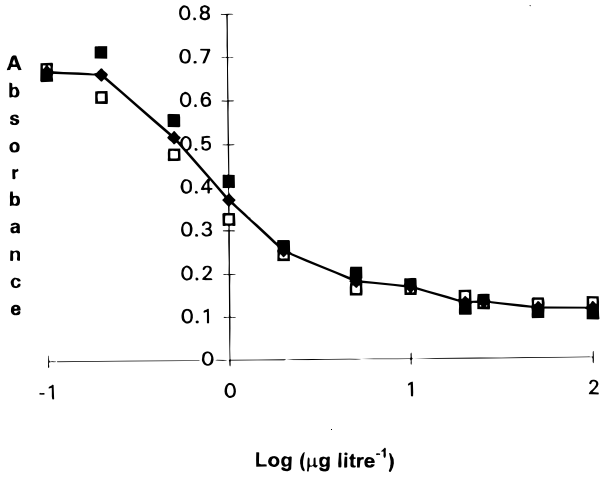

Fig. 1. Investigation of the linear response range with the 2,4-D ELISA kit: (■) first determination, (□) second determination, (◆) mean.

washed twice with water (25 ml), collected in a centrifuge tube, evaporated under nitrogen (to ~0·5 ml) and transferred quantitatively to a volumetric flask (1 ml). The extract was made up to volume with hexane prior to analysis by gas chromatography.

2.4.2 Gas chromatography-mass spectrometry (GC-MS)
GC-MS analysis was performed using a Hewlett Packard 5890 series II plus GC, coupled to a 5972 mass selective detector (MSD) and 7673 autosampler. A DB-1701 fused silica capillary column (30 m × 0·25 mm × 0·25 μm) was used with helium carrier gas at 8·8 psi. The detector temperature was set at 280°C and splitless injections (2 μl) were performed at 250°C. The oven was programmed as follows: 70°C for 1 min, then increasing at 40°C min^{-1} to 190°C, held for 2 min, then 10°C min^{-1} to 275°C and finally held for 1 min. The MSD was operated in selected ion monitoring (SIM) mode with a solvent delay of 5 min and dwell time of 150 ms. The ions monitored for 2,4-D methyl ester were *m/z* 175, 199 and 234. For 4-CPA (internal standard) ions at *m/z* 141 and 200 were monitored.

TABLE 1
Cross Reactivity Responses of the 2,4-D ELISA Kit

Compound	*Absorbance*[a,b]
Blank	0·712
2,4-D	0·544
2,4-D Na salt	0·540
2,4-D ethyl ester	0·328
2,4-D isopropyl ester	0·266
2,4,5-T	0·782

[a] Absorbance values are the means of duplicate measurements.
[b] Note that absorbance is inversely related to concentration of analyte present.

TABLE 2
Matrix Effect of Extracts of Orange on ELISA Response[a]

	Absorbance
Blank matrix	0·415
Blank matrix spiked at 1 μg $litre^{-1}$	0·310
Blank matrix at 10 × dilution	0·601
Blank matrix at 10 × dilution spiked at 1 μg $litre^{-1}$	0·386

[a] Absorbance values are the means of duplicate measurements.

3 RESULTS AND DISCUSSION

3.1 ELISA

A range of experimental parameters were investigated to determine the suitability of this kit as follows.

3.1.1 Linear range of the kit
Absorbance is inversely related to concentration. Figure 1 shows the calibration response of 2,4-D standards in water and demonstrates that the linear range is limited to 0·5–2·0 μg $litre^{-1}$ (−0·3 to + 0·3 on the log scale). This is much lower than the 0·5–100 μg $litre^{-1}$ suggested by the manufacturer but matches our previous experience with other kits. To use the kit in a semi-quantitative fashion, the 2,4-D concentration in the solutions applied to the wells must be close to 1 μg $litre^{-1}$ to fall within this linear range.

3.1.2 Cross reactivity
ELISA kits rely on the selectivity and specificity provided by the antibody-antigen relationship. However, it is often not the whole molecule that provides a response but the shape of part of it. Therefore, compounds of similar structure to 2,4-D may also generate a response. The cross reactivity of the kit was tested with standard solutions of structurally related compounds in water (1 μg $litre^{-1}$). Results are presented in Table 1.

At the 1 μg $litre^{-1}$ level, 2,4,5-T gave a response similar to the blank indicating that it is unlikely to interfere, even at relatively high concentrations. The sodium salt had, not surprisingly, a similar response to that of 2,4-D. The kit showed the greatest response to the ester compounds. As the nature of any residues of 2,4-D cannot be predicted, it is an advantage for screening purposes that the kit responds well to 2,4-D, the salt and esters. Calibration should be performed using 2,4-D itself which has the lowest sensitivity. This means that

esters, which have a higher response, will be overestimated but, most importantly, false negatives will be avoided.

3.1.3 Matrix effects

The kit was found to be very sensitive to matrix effects. Table 2 shows that changing the concentration of the matrix significantly alters the response and demonstrates the need for standards to be made up in blank matrix extracted and prepared in *exactly* the same way as the samples.

3.1.4 Dilution of methanol extracts with water

Methanol was selected as the extraction solvent. However, the antibodies in the ELISA kits do not work in high concentrations of organic solvent and the manufacturers suggest a maximum concentration of 10% methanol. Thus sample extracts which had been extracted with 100% methanol were diluted with water by a factor of at least 10 prior to application of the kit.

3.1.5 Repeatability data

To test the precision of the method, five aliquots of orange homogenate were spiked at 0·2 mg kg^{-1}, and five at 2·0 mg kg^{-1}; they were then extracted and measured using the kit. Results are presented in Table 3.

Repeatability was shown to be acceptable with clear and unequivocal differences between absorbance values of blanks and spiked samples. This allows the kit to be used as a screen to distinguish between reportable and blank samples, but not to quantify residues accurately.

TABLE 3
Repeatability of ELISA for Spiked Orange Extracts

Absorbance of spiked sample[a]		*Absorbance of blank*[a]
0·2 mg kg^{-1}	0·151	0·284
	0·164	0·288
	0·163	0·309
	0·177	0·262
	0·232	
Mean	0·177	0·286
r.s.d.[b]	18%	7%
2·0 mg kg^{-1}	0·170	0·555
	0·149	0·529
	0·146	0·581
	0·154	0·517
	0·176	
Mean	0·159	0·555
r.s.d.	8%	5%

[a] Absorbance values are the means of duplicate measurements.
[b] Relative standard deviation.

The difference in absorbances for the two sets of blanks (Table 3) was due to the matrix concentration of the blanks associated with the 2·0 mg kg^{-1} samples, being one-tenth that of the 0·2 mg kg^{-1} samples and corresponding blanks.

3.1.6 Edge effects of ELISA microplates

The wells are arranged in horizontal strips of 12, eight strips deep. Occasionally a sample in a well on the edge of a strip gave a response noticeably different to that with a duplicate sample in the well immediately inside. This discrepancy did not appear to be related to the plate reader. Edge effects were once a well-known phenomenon in assays of this nature, but the manufacturers claim to have eliminated this problem. This evidence suggests that edge effects have not been wholly eradicated. If edge well problems are suspected then only the wells in the middle should be used.

3.2 Chromatographic method

The second area of work was to develop a simple confirmatory method for analysing 2,4-D residues in oranges using gas chromatography-mass spectrometry.

3.2.1 Methylation

To use gas chromatography (GC), 2,4-D must be derivatised to reduce its polarity. Esterification using BF_3 in methanol is a well-known procedure for derivatising carboxylic acids to methyl esters.[5] It proved possible, in a single step, to methylate 2,4-D to the methyl ester and to transesterify 2,4-D esters to the methyl ester, thereby removing the need to hydrolyse 2,4-D esters prior to derivatisation. The filtered methanol extract of the oranges could be derivatised directly without cleanup. Recovery data for the derivatisation of 2,4-D and its isopropyl ester spiked into a methanol extract of orange ranged from 98 to 106% and from 98 to 120% at 0·2 and 2·0 mg kg^{-1} levels, respectively so that derivatisation was quantitative.

3.2.2 Internal standard

The optimum choice of internal standard was an isotopically labelled analogue of 2,4-D, e.g. the ring D_3 or ring ^{13}[C]-analogues; however, the high cost of these compounds (£481 for 5 mg and £445 for 1 mg respectively) ruled them out. Second choice was a dihalogen homologue but neither 2,4-difluoro- or 2,4-dibromophenoxyacetic acid was available commercially. Third choice was another chlorophenoxyacetic acid, and two such compounds, 4-CPA and 2,4,5-T, were available. Both are used as pesticides, but 4-CPA is

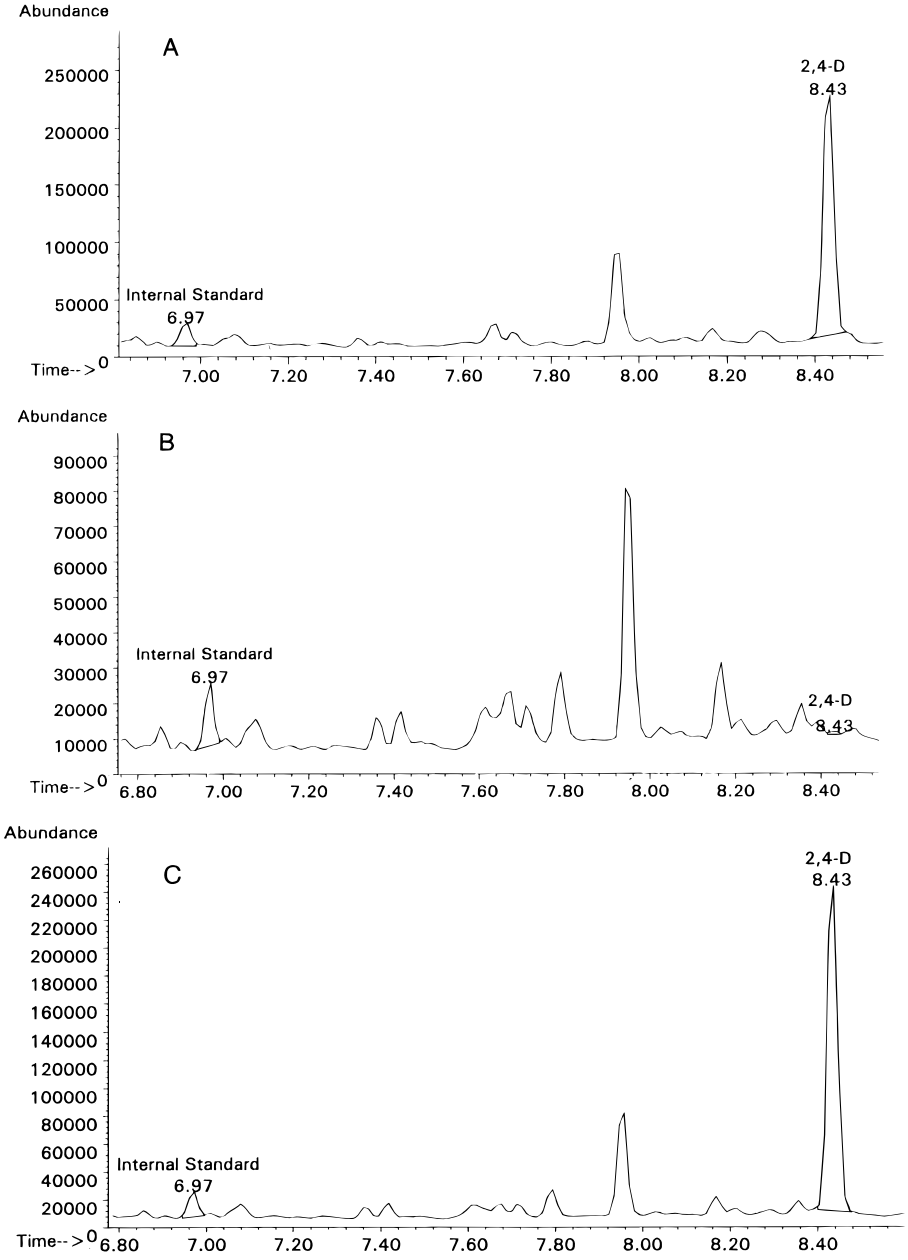

Fig. 2. Total ion chromatograms (TIC) of the ions monitored in the GC-MS procedure. A, Orange extract spiked with 1 mg kg^{-1} 2,4-D. B, Blank orange extract. C, Whole orange extracted spiked with 1 mg kg^{-1} 2,4-D.

more restricted in its application, being used only to set tomato blossom and inhibit sprout formation of mung beans. There is a small risk that it may be present in the oranges through misuse or, as a structurally related compound, that it may occur as a contaminant in 2,4-D formulations; however, to date, we have found no evidence of 4-CPA being present in any of the oranges analysed.

3.2.3 Method performance

The method was validated by spiking orange pulp with 2,4-D or 2,4-D isopropyl ester at 0·2, 1·0 and 2·0 mg kg^{-1} (Table 4 and Fig. 2). Good recoveries were obtained for both compounds.

3.3 Comparison of effort for the two methods

Less than half the staff time (9 compared with 19 h) was required for the analysis of 20 samples by the ELISA method compared to the GC-MS method (Table 5).

3.4 Analysis of retail samples

It was impossible to obtain samples known to contain incurred residues. Therefore, both methods were used to

TABLE 4
Recoveries of 2,4-D from Spiked Homogenised Whole Oranges by the GC-MS Method

	Recovery (%)[a] sample spiked with (mg kg^{-1})		
	0·2	*1·0*	*2·0*
2,4-D	85	120	115
	90	120	100
	—[b]	110	75
Mean	88	117	97
2,4-D isopropyl ester	95	107	94
	95	112	85
	85	99	85
Mean	92	106	88

[a] Results are the means of duplicate measurements.
[b] Sample lost.

TABLE 6
Retail Samples Analysed for 2,4-D Residues

Country of origin	*Residue level (mg kg^{-1})*	
	ELISA	*GC-MS*
Israel	12	0·28
Israel	11	0·42
Israel	5	0·30
Israel	4	0·32
Unknown	<0·2	na[a]
Unknown	<0·2	na
South Africa	<0·2	na
Spain	<0·2	<0·2
Cyprus	<0·2	na
Unknown	<0·2	na

[a] na = not analysed.

analyse a small survey (10 samples) of oranges on retail sale. Results are presented in Table 6. Four of the samples were positive using the ELISA kit. These four, plus one negative sample were re-analysed by GC-MS.

Quantitative agreement between the methods was very poor owing to the ELISA technique possessing a very limited linear range. However, it can be seen from Table 6 that the ELISA performed excellently as a screening technique. All four samples which yielded positive responses by ELISA were confirmed by GC-MS.

4 CONCLUSIONS

From the results of this investigation, it is clear that the commercially available ELISA kit can be used successfully for screening for 2,4-D residues in oranges. Use of this kit has great economic advantage in terms of staff effort.

The GC-MS method successfully complements this screening procedure and it also has considerable advantages over published methods in that the very efficient methylation and trans-esterification process obviates the need for additional clean-up or hydrolysis steps.

TABLE 5
Comparison of Staff Time Required for the Analysis of 20 Samples

	ELISA (h)	*GC-MS (h)*
Extraction	6	6
Derivatisation		9[a]
Measurement/data handling	3	4[b]
Total time for 20 samples	9	19

[a] Excludes samples standing overnight.
[b] Excludes GC-MS runtime.

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